

Evolution of Resistance to Diclofop-methyl in Ryegrass (*Lolium multiflorum*): Investigation of the Role of Introgression with Related Species*

María A. Martínez-Ghersa,^{1†} Claudio M. Ghersa,¹ Martín M. Vila-Aiub,¹ Emilio H. Satorre² & Steve R. Radosevich³

¹ Depto. de Ecología Facultad de Agronomía, Universidad de Buenos Aires, 1417-Buenos Aires, Argentina

² Depto. de Producción Vegetal, Facultad de Agronomía, Universidad de Buenos Aires, 1417-Buenos Aires, Argentina

³ Dept. of Forest Science, Oregon State University, 97331, USA

(Received 16 May 1997; revised version received 26 June 1997; accepted 15 July 1997)

Abstract: Populations of *Lolium multiflorum* (ryegrass) exposed to increasing levels of herbicide selection have variability in the rate of evolution of herbicide resistance. We tested the hypothesis that herbicide dose and gene flow with related species are important factors regulating the rate of evolution of herbicide resistance in *L. multiflorum*. Seeds of the latter were planted in pure stands and in mixture with *Festuca rubra* and subjected to four herbicide rates. The level of herbicide resistance attained by the offspring after two years of selection was evaluated. Evolution of resistance observed in the field was compared to that calculated by Gressel and Segel's rotational model.

Pestic. Sci., 51, 305–308, 1997

No. of Figures: 1. No. of Tables: 2. No. of Refs: 17

Key words: ryegrass, diclofop, resistance, introgression, *Festuca*

1 INTRODUCTION

Italian ryegrass (*Lolium multiflorum* Lam.) is cultivated as a forage and turf grass species in many temperate regions of the world. However, it is also a serious weed in wheat and barley crops, causing important yield losses in the USA and Australia. Cultural tillages and herbicides are used to control the weed.

Italian ryegrass has developed an inheritable resistance to the selective herbicide diclofop-methyl.¹ The traditional assumption is that resistance is introduced by a point mutation, which is afterwards selected by

continuous use of herbicides.^{2–4} Italian ryegrass populations differ in the level of resistance. The existence of ryegrass populations that stayed susceptible after being exposed to high doses of herbicide, and the existence of populations that developed different rates of resistance when exposed to the same herbicide selection pressure have been reported.³ This information suggests that there could be more than just a single mechanism related to the evolution of resistance in ryegrass populations, or different point mutations conferring differing resistance intensity and cross-resistance patterns.⁵ Genetic introgression (through gene flow) with genetically related resistant species^{6,7} and somatic variations that are environmentally induced⁷ have been suggested as possible alternative mechanisms.

Some species of *Festuca* and *Vulpia* common to the native and agricultural flora where ryegrass grows are tolerant to diclofop-methyl.⁶ DNA analysis in chloroplasts has confirmed that *L. multiflorum* is related

* Based on a presentation at the Conference 'Resistance '97—Integrated Approach to Combating Resistance' organised by the Institute of Arable Crops Research in collaboration with the SCI Pesticides Group and the British Crop Protection Council and held at the Institute of Arable Crops Research, Harpenden, Herts, UK on 14–16 April 1997.

† To whom correspondence should be addressed.

phylogenetically to the species of these genera, so that they may thus be considered as a common gene pool.^{8,9} There is little structural differentiation between chromosomes of members of these genera, and there are no effective isolation barriers to gene flow among many of the species.¹⁰ *L. multiflorum* and *Festuca rubra* L. are allogamous. Hybrids between the two species have been found to occur both naturally and through intentional crossing.¹¹

Therefore, we used simulation techniques and set up a field experiment to evaluate the role of gene flow between *L. multiflorum* and a related species, naturally resistant to diclofop-methyl, in the evolution of a resistant population of *L. multiflorum* in different ecological scenarios.

2 EXPERIMENTAL METHODS

2.1 Simulation experiment

The rotational model proposed by Gressel and Segel¹² was used to calculate changes in resistance level of a ryegrass population during 10 years of repeated herbicide application at three different rates, half, whole and twice that recommended on the label. Simulation was carried out for a ryegrass population growing in pure stand and in admixture with a related species resistant to diclofop-methyl. We assumed an initial frequency (N_0) of 1×10^{-6} R individuals¹² for the pure stand ryegrass population. For simulation purposes we assessed the effect of introgression with the related species, modifying the initial frequency of the resistant gene to be 1×10^{-4} .¹¹ Values for α (overall selection pressure) were obtained in preliminary experiments in which a ryegrass population was sprayed with one half (0.56 kg ha^{-1}), full (1.12 kg ha^{-1}), and twice (2.24 kg ha^{-1}) the label rate of diclofop-methyl ('Hoelon') and registered $\alpha_r = 0.91$, and $\alpha_s = 0.06$ for the half and full rates, respectively, and $\alpha_s = 0.01$ for twice the label rate. Values for the other parameters (f , fitness of the resistant and n , average residence time in the soil seed bank) were obtained from the literature.^{12–14} Previous studies showed no evidence that herbicide-resistant ryegrass plants are any less fit than susceptible ones in wheat fields.¹⁴ However, we considered the possibility that individuals coming up from introgression could be less fit than true *Lolium* (i.e. reduced pollen production and viability, or seed size).¹⁰

2.2 Field experiment

A long-term field experiment was laid out to evaluate changes in the resistance level of a ryegrass population. Susceptible ryegrass seeds were sown in pure stands and in admixture with *F. rubra* in 40-m^2 plots separated by

2-m wide roads. The latter was chosen as the potential donor for resistant genes because there is abundant information about it⁷ and it is not naturalized in the Pampa. Hence, any part of its DNA eventually identified in the ryegrass plants could only be a result of our experiment. Pure and mixed stand plots were randomly assigned to a gradient of diclofop-methyl rates (0, 0.5, 1 and $2 \times$ label), with which plants were sprayed each growing season. Treatment plots were replicated four times. Ryegrass seeds were collected from a natural population in a grassland of the Pampa, Argentina ($34^\circ 06'S$ and $60^\circ 25'W$) that had never been subjected to herbicide application. In order to reduce pollen flow among plots, wind barriers were built by sowing rye in the roads, and plants were covered with a frost protection net during the flowering period.

To determine the level of resistance (R) attained after two years of herbicide application, seeds harvested from each plot were germinated in pots and sprayed at the two leaves stage with diclofop-methyl (1.12 kg ha^{-1}) or left unsprayed. Seedlings were counted 29 days after spraying, and the dry weight of survivors was also registered. The proportion of R plants in the population was estimated as the proportion of individuals attaining a dry weight $> 50 \text{ mg}$ (minimum dry weight recorded for control individuals).

3 RESULTS AND DISCUSSION

In the simulation experiments, the model proposed by Gressel and Segel¹² predicts that a detectable population of resistant individuals should appear after three years of herbicide application, under the worst scenario (with introgression of resistance genes and herbicide rate twice the label) (Fig. 1c). Under high selection pressure ($2 \times$) a hundred-fold difference in initial gene frequency (as could result from introgression) translates into only one generation difference in the time it takes for a population to become predominantly resistant (Fig. 1c). That difference increases when selection pressure is lowered (Figs 1a & b). Evolution of resistance would be delayed by as much as five years if the rate was reduced to label or lower, and introgression was absent (Figs 1a & b). Reducing the fitness of the resistant population by 20% did not change the period of undetectable resistance in any of the three scenarios explored (Fig. 1).

In the field experiment, the frequency of resistant individuals in the plots after two years of herbicide application was already high enough to allow for the detection of differences in the level of resistance reached in the different treatments (Table 1). As predicted by the model, the highest resistance level in both the pure stand and mixed plots corresponded to the treatments with the selection pressure exerted by twice the label rate (Table 1). In the plots with *Festuca*, a similar resist-

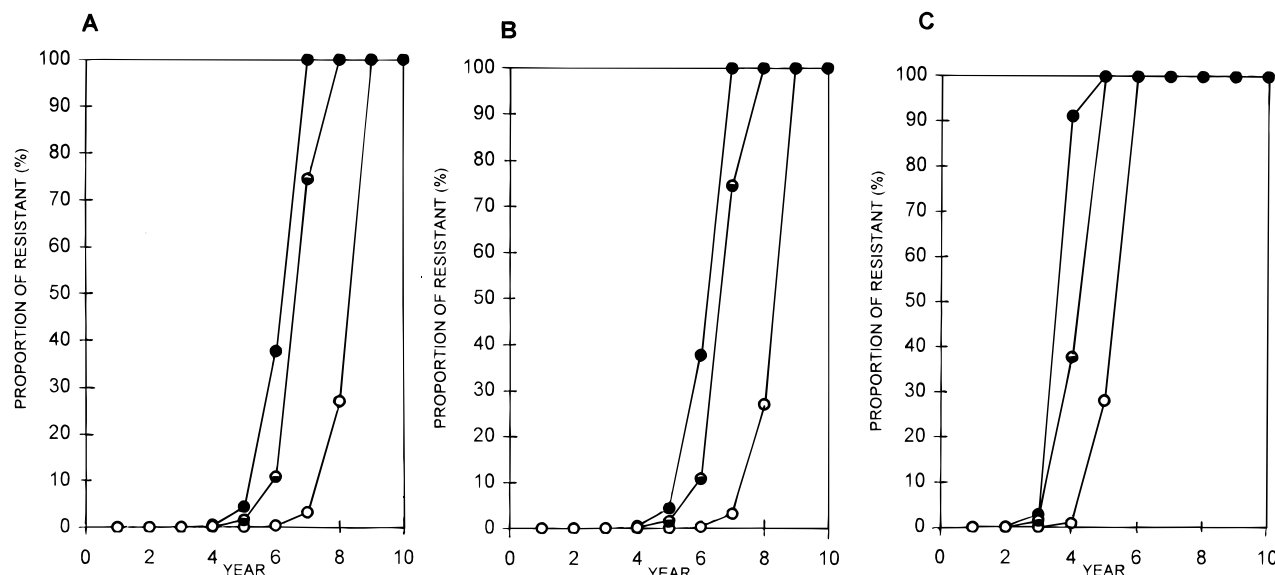


Fig. 1. Evolution of resistance in a ryegrass population when growing (○) alone or (●) in mixture with *Festuca* for different herbicide dose selection pressures, calculated with Gressel and Segel's rotational model.¹² (◐) Effect of reducing *f* value (fitness of resistants) to 0.8. See Section 2.1 for values assigned to model parameters.

ance level was attained when plants received half the herbicide rate every year. This was evidenced in the proportion of individuals surviving herbicide application, with no evidence of growth inhibition (> 50 mg), and also in the mean weight of survivors (Table 2). Previous studies showed that resistant and susceptible ryegrass individuals have similar growth rates in herbicide-free conditions and a wide range of densities.¹⁴ Therefore the observed differences in weight were a response to herbicide application.

Our data suggest that the Pampa ryegrass population could rapidly become resistant under herbicide selection pressure, as has occurred with other annual ryegrass populations in the USA and Australia.^{1,3} Differences between the initial frequency of resistant genes in the simulated and sown populations might explain the different rates of development of resistance found with the

experiments. Other authors have reported initial frequencies of resistant individuals as high as 2% in populations of *L. rigidum* Gaud. in Australia with no history of herbicide use.¹⁵ Gene exchange with related species could be responsible for a high frequency of 'background' resistance. *L. multiflorum* has been found to coexist with species of the genera *Vulpia*, resistant to diclofop-methyl, in the Pampas grassland.¹⁶ To our knowledge, there are no studies showing hybridization rates between these two species. However, our data from the field experiment suggest that, in the mixed plots where the herbicide selection pressure was low (half-rate application), introgression might have accelerated the evolution of a resistant ryegrass population.

In spite of the fact that we are still missing the critical evidence produced by a gene map to prove unequivocally that the increase in the rate of evolution observed in our plots with mixed stands is caused by gene flow,

TABLE 1

Average Proportion of Ryegrass Seedlings Surviving Treatment with Diclofop-methyl^{a,b,c}

Herbicide rate ^d in seed field plots	Ryegrass seedlings surviving treatment (%)	
	Pure stand	With Festuca
0 ×	0	0
0.5 ×	0	6
1 ×	2	3
2 ×	14	8

^a Applied at 1.12 kg ha⁻¹.

^b Seedlings weighing less than 50 mg were not considered.

^c Seeds for the bioassays were harvested from field plots after two years of repeated herbicide application at different rates.

^d × : label rate.

TABLE 2

Effect of Application of Diclofop-methyl^a on the Mean Dry Weight of Survivors of Ryegrass.^b

Herbicide rate ^c in seed field plots	Mean dry weight of survivors (mg)	
	Pure stand	With Festuca
0 ×	11.6	11.4
0.5 ×	11.4	27.8
1 ×	24.9	32.3
2 ×	53.2	35.8

^a Applied at 1.12 kg ha⁻¹.

^b Seeds for the bioassays were harvested from field plots after two years of repeated herbicide application at different rates.

^c × : label rate.

data from these two first years of field experiment strongly support the idea that gene flow among related species of ryegrass is involved in the acceleration of the evolution of herbicide resistance, particularly when we consider low selective pressures. In natural systems, this type of mechanism involving gene flow under reduced selection pressure has already been documented for the development of resistance to plant toxins in insect populations.¹⁷

Knowledge of the factors that maintain variability in traits conferring resistance to herbicides in weed populations is critical for managing resistance. For example, management strategies could be quite different if the resistant allele is rare or initially common.¹⁵ However, studies should not be kept at the molecular level, since population structure is important in determining how mutations are spread or maintained. Without further studies on the population ecology of resistant and susceptible populations, that include at least qualitative information on the level of gene flow between populations, there is little hope of understanding why one species develops resistance and others do not.

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